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(54) Title: FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF (57) Abstract The present invention is directed to novel fluorescent proteins from non-bioluminescent organisms from the Class Anthozoa. Also disclosed are methods of identifying nucleic acid sequence encoding the fluorescent proteins and further analyzing the proteins.		

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**FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES
OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND
USES THEREOF**

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10

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of molecular biology. More specifically, this invention relates to novel fluorescent proteins, 15 methods of identifying the DNA sequences encoding the proteins and uses thereof.

Description of the Related Art

Fluorescence labeling is a particularly useful tool for 20 marking a protein, cell, or organism of interest. Traditionally, a protein of interest is purified, then covalently conjugated to a fluorophore derivative. For *in vivo* studies, the protein-dye complex is then inserted into cells of interest using micropipetting or a method of reversible permeabilization. The dye attachment and insertion steps, 25 however, make the process laborious and difficult to control. An alternative method of labeling proteins of interest is to concatenate or fuse the gene expressing the protein of interest to a gene expressing a marker, then express the fusion product. Typical markers for this method of protein labeling include β -galactosidase, firefly luciferase

and bacterial luciferase. These markers, however, require exogenous substrates or cofactors and are therefore of limited use for *in vivo* studies.

5 A marker that does not require an exogenous cofactor or substrate is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a protein with an excitation maximum at 395 nm, a second excitation peak at 475 nm and an emission maximum at 510 nm. GFP is a 238-amino acid protein, with amino acids 65-67 involved in the formation of the chromophore.

10 Uses of GFP for the study of gene expression and protein localization are discussed in detail by Chalfie et al. in *Science* 263 (1994), 802-805, and Heim et al. in *Proc. Nat. Acad. Sci.* 91 (1994), 12501-12504. Additionally, Rizzuto et al. in *Curr. Biology* 5 (1995), 635-642, discuss the use of wild-type GFP as a tool for visualizing
15 subcellular organelles in cells, while Kaether and Gerdes in *Febs Letters* 369 (1995), 267-271, report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng in *Febs Letters* 369 (1995), 331-334, while GFP expression in *Drosophila* embryos is described by
20 Davis et al. in *Dev. Biology* 170 (1995), 726-729.

Crystallographic structures of wild-type GFP and the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel (Ormö et al., *Science* 273 (1996), 1392-1395; Yang, et al., *Nature Biotechnol* 14 (1996), 1246-1251). The barrel consists of beta sheets
25 in a compact structure, where, in the center, an alpha helix containing the chromophore is shielded by the barrel. The compact structure makes GFP very stable under diverse and/or harsh conditions such as protease treatment, making GFP an extremely useful reporter in

general. However, the stability of GFP makes it sub-optimal for determining short-term or repetitive events.

A great deal of research is being performed to improve the properties of GFP and to produce GFP reagents useful and optimized for a variety of research purposes. New versions of GFP have been developed, such as a "humanized" GFP DNA, the protein product of which has increased synthesis in mammalian cells (Haas, et al., *Current Biology* 6 (1996), 315-324; Yang, et al., *Nucleic Acids Research* 24 (1996), 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue-, cyan- and yellow-green light emitting versions. Despite the great utility of GFP, however, other fluorescent proteins with properties similar to or different from GFP would be useful in the art. Novel fluorescent proteins result in possible new colors, or produce pH-dependent fluorescence. Other benefits of novel fluorescent proteins include fluorescence resonance energy transfer (FRET) possibilities based on new spectra and better suitability for larger excitation.

The prior art is deficient in novel fluorescent proteins wherein the DNA coding sequences are known. The present invention fulfills this long-standing need in the art.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence
5 encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is
10 provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid
15 sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a
20 fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the
25 nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an

intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

10 **Figure 1** shows the modified strategy of 3'-RACE used to isolate the target fragments. Sequences of the oligonucleotides used are shown in Table 2. Dp1 and Dp2 are the degenerate primers used in the first and second PCR, respectively (see Tables 3 and 4 for the sequences of degenerate primers).

15 **Figure 2A** shows multiple alignment of novel fluorescent proteins. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP). Two proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming beta-sheets are underlined; the residues whose side chains form the interior of the beta-can are shaded (according to Yang et al., *Nature Biotechnol.* 14, 1246-1251 (1996)).
20 **Figure 2B** shows the N-terminal part of cFP484, which has no
25 homology with the other proteins. The putative signal peptide is underlined.

Figure 3 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia majano*, amFP486.

Figure 4 shows the excitation and emission spectrum of the novel fluorescent protein from *Clavularia*, cFP484.

Figure 5 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP506.

5 Figure 6 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP538.

Figure 7 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma striata*, dsFP483.

10 Figure 8 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, drFP583.

Figure 9 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia sulcata*, asFP600.

Figure 10 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dgFP512.

15 Figure 11 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dmFP592.

DETAILED DESCRIPTION OF THE INVENTION

20 As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *Aequorea victoria* GFP (SEQ ID No. 54) has been disclosed in Prasher et al., *Gene* 111 (1992), 229-33.

25 As used herein, the term "EGFP" refers to mutant variant of GFP having two amino acid substitutions: F64L and S65T (Heim et al., *Nature* 373 (1995), 663-664). The term "humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for

expression of the protein in human cells (Yang et al., *Nucleic Acids Research* 24 (1996), 4592-4593).

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells and Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

15 A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

25 A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3'

(carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination
5 sequence may be located 3' to the coding sequence.

As used herein, the term "hybridization" refers to the process of association of two nucleic acid strands to form an antiparallel duplex stabilized by means of hydrogen bonding between residues of the opposite nucleic acid strands.

10 The term "oligonucleotide" refers to a short (under 100 bases in length) nucleic acid molecule.

"DNA regulatory sequences", as used herein, are transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that
15 provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining
20 the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a
25 transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by
5 exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to
10 eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter
15 cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of the DNA construct is an
20 identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example,
25 heterologous DNA includes coding sequence in a construct where portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "reporter gene" refers to a coding sequence attached to heterologous promoter or enhancer elements and whose product may be assayed easily and quantifiably when the construct is introduced into tissues or cells.

5 The amino acids described herein are preferred to be in the "L" isomeric form. The amino acid sequences are given in one-letter code (A: alanine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophane; Y: tyrosine; X: any
10 residue). NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243 (1969), 3552-
15 59 is used.

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

20 In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence encodes a peptide having a sequence selected from the group
25 consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a

fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid
5 sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a
10 fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the
15 nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an
20 intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1Biological Material

- 5 Novel fluorescent proteins were identified from several genera of Anthozoa which do not exhibit any bioluminescence but have fluorescent color as observed under usual white light or ultraviolet light. Six species were chosen (see Table 1).

10

TABLE 1Anthozoa Species Used in This Study

Species	Area of Origination	Fluorescent Color
Anemonia majano	Western Pacific	bright green tentacle tips
Clavularia sp.	Western Pacific	bright green tentacles and oral disk
Zoanthus sp.	Western Pacific	green-yellow tentacles and oral disk
Discosoma sp. "red"	Western Pacific	orange-red spots oral disk
Discosoma striata	Western Pacific	blue-green stripes on oral disk
Discosoma sp. "magenta"	Western Pacific	faintly purple oral disk
Discosoma sp.	Western Pacific	green spots on oral disk

"green"		
Anemonia sulcata	Mediterranean	purple tentacle tips

EXAMPLE 2cDNA Preparation

Total RNA was isolated from the species of interest according to the protocol of Chomczynski and Sacchi (Chomczynski P., et al., *Anal. Biochem.* 162 (1987), 156-159). First-strand cDNA was synthesized starting with 1-3 µg of total RNA using SMART PCR cDNA synthesis kit (CLONTECH) according to the provided protocol with the only alteration being that the "cDNA synthesis primer" provided in the kit was replaced by the primer TN3 (5'- CGCAGTCGACCG(T)₁₃, SEQ ID No. 1) (Table 2). Amplified cDNA samples were then prepared as described in the protocol provided except the two primers used for PCR were the TS primer (5'-AAGCAGTGGTATCAACGCAGAGT, SEQ ID No. 2) (Table 2) and the TN3 primer (Table 2), both in 0.1 µM concentration.

Twenty to twenty-five PCR cycles were performed to amplify a cDNA sample. The amplified cDNA was diluted 20-fold in water and 1 µl of this dilution was used in subsequent procedures.

TABLE 2

Oligos Used in cDNA Synthesis and RACE

5 TN3: 5'-CGCAGTCGACCG(T)₁₃
(SEQ ID No. 1)

T7-TN3: 5'-GTAATACGACTCACTATAGGGCCGCGAGTCGACCG(T)₁₃
(SEQ ID No. 17)

10 TS-primer: 5'-AAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 2)

T7-TS:
15 5'-GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 18)

T7: 5'-GTAATACGACTCACTATAGGGC
(SEQ ID No. 19)

20 TS-oligo 5'-AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG
(SEQ ID No. 53)

EXAMPLE 3Oligo Design

To isolate fragments of novel fluorescent protein cDNAs,
5 PCR using degenerate primers was performed. Degenerate primers
were designed to match the sequence of the mRNAs in regions that
were predicted to be the most invariant in the family of fluorescent
proteins. Four such stretches were chosen (Table 3) and variants of
degenerate primers were designed. All such primers were directed to
10 the 3'-end of mRNA. All oligos were gel-purified before use. Table 2
shows the oligos used in cDNA synthesis and RACE.

TABLE 3

Key Amino Acid Stretches and Corresponding Degenerate Primers Used for Isolation of Fluorescent Proteins

5

Stretch Position according to A. victoria GFP (7)	Amino Acid Sequence of the Key Stretch	Degenerated Primer Name and Sequence
20-25	GXVNGH (SEQ ID No. 3)	NGH: 5'- GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA (SEQ ID No. 4)
31-35	GEGEG (SEQ ID No. 5) GEGNG (SEQ ID No. 8)	GEGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG (SEQ ID No. 6) GEGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG (SEQ ID No. 7) GNGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG (SEQ ID No. 9) GNGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG (SEQ ID No. 10)
127-131	GMNFP (SEQ ID No. 11) GVNFP (SEQ ID No. 12)	NFP: 5' TTC CA(C,T) GGT (G,A)TG AA(C,T) TT(C,T) CC (SEQ ID NO. 13)
134-137	GPVM (SEQ ID No. 14)	PVMa: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG (SEQ ID NO. 15) PVMb: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG (SEQ ID NO. 16)

EXAMPLE 4Isolation of 3'-cDNA Fragments of nFPs

The modified strategy of 3'-RACE was used to isolate the
5 target fragments (see Figure 1). The RACE strategy involved two
consecutive PCR steps. The first PCR step involved a first degenerate
primer (Table 4) and the T7-TN3 primer (SEQ ID No. 17) which has a 3'
portion identical to the TN3 primer used for cDNA synthesis (for
sequence of T7-TN3, Table 2). The reason for substituting the longer
10 T7-TN3 primer in this PCR step was that background amplification
which occurred when using the shorter TN3 primer was suppressed
effectively, particularly when the T7-TN3 primer was used at a low
concentration (0.1 μ M) (Frohman et al., (1998) *PNAS USA*, 85, 8998-
9002). The second PCR step involved the TN3 primer (SEQ ID No. 1,
15 Table 2) and a second degenerate primer (Table 4).

TABLE 4

Combinations of Degenerate Primers for First and Second PCR Resulting in Specific Amplification of 3'-Fragments of nFP cDNA

Species	First Degenerate Primer	Second Degenerate Primer
Anemonia majano	NGH (SEQ ID No. 4)	GNGb (SEQ ID No. 10)
Clavularia sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Zoanthus sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Discosoma sp. "red"	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6), NFP (SEQ ID No. 13) or PVMb (SEQ ID No. 16)
Discosoma striata	NGH (SEQ ID No. 4)	NFP (SEQ ID No. 13)
Anemonia sulcata	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6) or NFP (SEQ ID No. 13)

5

The first PCR reaction was performed as follows: 1 µl of 20-fold
 10 dilution of the amplified cDNA sample was added into the reaction
 mixture containing 1X Advantage KlenTaq Polymerase Mix with
 provided buffer (CLONTECH), 200 µM dNTPs, 0.3 µM of first degenerate

primer (Table 4) and 0.1 μ M of T7-TN3 (SEQ ID No. 17) primer in a total volume of 20 μ l. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C, 1 min.; 72°C, 40 sec; 24 cycles for 95°C, 10 sec.; 62°C, 30 sec.; 72°C, 40 sec. The reaction was then diluted 20-fold in water and 1 μ l of this dilution was added to a second PCR reaction, which contained 1X Advantage KlenTaq Polymerase Mix with the buffer provided by the manufacturer (CLONTECH), 200 μ M dNTPs, 0.3 μ M of the second degenerate primer (Table 4) and 0.1 μ M of TN3 primer. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C (for GEG/GNG or PVM) or 52°C (for NFP), 1 min.; 72°C, 40 sec; 13 cycles for 95°C, 10sec.; 62°C (for GEG/GNG or PVM) or 58°C (for NFP), 30 sec.; 72°C, 40 sec. The product of PCR was cloned into PCR-Script vector (Stratagene) according to the manufacturer's protocol.

Different combinations of degenerate primers were tried in the first and second PCR reactions on the DNA from each species until a combination of primers was found that resulted in specific amplification--meaning that a pronounced band of expected size (about 650-800 bp for NGH and GEG/GNG and 350-500 bp for NFP and PVM--sometimes accompanied by a few minor bands) was detected on agarose gel after two PCR reactions. The primer combinations of choice for different species of the Class Anthozoa are listed in Table 4. Some other primer combinations also resulted in amplification of fragments of correct size, but the sequence of these fragments showed no homology to the other fluorescent proteins identified or to *Aequorea victoria* GFP.

EXAMPLE 5Obtaining Full-Length cDNA Copies

Upon sequencing the obtained 3'-fragments of novel
5 fluorescent protein cDNAs, two nested 5'-directed primers were
synthesized for cDNA (Table 5), and the 5' ends of the cDNAs were
then amplified using two consecutive PCRs. In the next PCR reaction,
the novel approach of "step-out PCR" was used to suppress background
amplification. The step-out reaction mixture contained 1x Advantage
10 KlenTaq Polymerase Mix using buffer provided by the manufacturer
(CLONTECH), 200 μ M dNTPs, 0.2 μ M of the first gene-specific primer
(see Table 5), 0.02 μ M of the T7-TS primer (SEQ ID No. 18), 0.1 μ M of
T7 primer (SEQ ID No. 19) and 1 μ l of the 20-fold dilution of the
amplified cDNA sample in a total volume of 20 μ l. The cycling profile
15 was (Hybaid OmniGene Thermocycler, tube control mode): 23-27
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of
amplification was diluted 50-fold in water and one μ l of this dilution
was added to the second (nested) PCR. The reaction contained 1X
Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH),
20 200 μ M dNTPs, 0.2 μ M of the second gene-specific primer and 0.1 μ M
of TS primer (SEQ ID No. 2) in a total volume of 20 μ l. The cycling
profile was (Hybaid OmniGene Thermocycler, tube control mode): 12
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of
amplification was then cloned into pAtlas vector (CLONTECH) according
25 to the manufacturer's protocol.

TABLE 5Gene-Specific Primers Used for 5'-RACE

Species	First Primer	Second (Nested) Primer
Anemonia majano	5'-GAAATAGTCAGGCATACTGGT (SEQ ID No. 20)	5'-GTCAGGCATAC TGGTAGGAT (SEQ ID No. 21)
Clavularia sp.	5'-CTTGAAATAGTCTGCTATATC (SEQ ID No. 22)	5'-TCTGCTATATC GTCTGGGT (SEQ ID No. 23)
Zoanthus sp.	5'- GTTCTTGAAATAGTCTACTATGT (SEQ ID No. 24)	5'-GTCTACTATGTCTT GAGGAT (SEQ ID No. 25)
Discosoma sp. "red"	5'-CAAGCAAATGGCAAAGGTC (SEQ ID No. 26)	5'-CGGTATTGTGGCC TTCGTA (SEQ ID No. 27)
Discosoma striata	5'-TTGTCTTCTTCTGCACAAC (SEQ ID No. 28)	5'-CTGCACAACGG GTCCAT (SEQ ID No. 29)
Anemonia sulcata	5'-CCTCTATCTTCATTTCCTGC (SEQ ID No. 30)	5'-TATCTTCATTTCCT GCGTAC (SEQ ID No. 31)
Discosoma sp. "magenta"	5'-TTCAGCACCCCATCACGAG (SEQ ID No. 32)	5'-ACGCTCAGAGCTG GGTTCC (SEQ ID No. 33)
Discosoma sp. "green"	5'-CCCTCAGCAATCCATCACGTTC (SEQ ID No. 34)	5'-ATTATCTCAGTGGA TGGTTC (SEQ ID No. 35)

EXAMPLE 6

Expression of nFPs in *E. coli*

5 To prepare a DNA construct for novel fluorescent protein expression, two primers were synthesized for each cDNA: a 5'-directed "downstream" primer with the annealing site located in the 3'-UTR of the cDNA and a 3'-directed "upstream" primer corresponding to the site of translation start site (not including the first ATG codon) (Table 10 6). Both primers had 5'-heels coding for a site for a restriction endonuclease; in addition, the upstream primer was designed so as to allow the cloning of the PCR product into the pQE30 vector (Qiagen) in such a way that resulted in the fusion of reading frames of the vector-encoded 6xHis-tag and nFP. The PCR was performed as follows: 1 µl of 15 the 20-fold dilution of the amplified cDNA sample was added to a mixture containing 1x Advantage KlenTaq Polymerase Mix with buffer provided by the manufacturer (CLONTECH), 200 µM dNTPs, 0.2 µM of upstream primer and 0.2 µM of downstream primer, in a final total volume of 20 µl. The cycling profile was (Hybaid OmniGene 20 Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of this amplification step was purified by phenol-chlorophorm extraction and ethanol precipitation and then cloned into pQE30 vector using restriction endonucleases corresponding to the primers' sequence according to standard 25 protocols.

All plasmids were amplified in XL-1 blue *E. coli* and purified by plasmid DNA miniprep kits (CLONTECH). The recombinant clones were selected by colony color, and grown in 3 ml of LB medium (supplemented with 100 µg/ml of ampicillin) at 37°C overnight. 100 µl

of the overnight culture was transferred into 200 ml of fresh LB medium containing 100 µg/ml of ampicillin and grown at 37°C, 200 rpm up to OD₆₀₀ 0.6-0.7. 1 mM IPTG was then added to the culture and incubation was allowed to proceed at 37°C for another 16 hours. The
5 cells were harvested and recombinant protein, which incorporated 6x His tags on the N-terminus, was purified using TALON™ metal-affinity resin according to the manufacturer's protocol (CLONTECH).

TABLE 6

Primers Used to Obtain Full Coding Region of nEPs for Cloning into Expression Construct

Species	Upstream Primer	Downstream Primer
Anemonia majano	5' -acatggatccgctctttcaaaca agtttacc (SEQ ID No. 36) BamHI	5'-tagtactcgcgagcttattcgta tttcagtgaatc (SEQ ID No. 37) XhoI
Clavularia sp.	L: 5'-acatggatccaacattttttga gaaacg (SEQ ID No. 38) BamHI S: 5'-acatggatccaaagctctaacc accatg (SEQ ID No. 39) BamHI	5'-tagtactcgcgagcaacacaa accctcagacaa (SEQ ID No. 40) XhoI
Zoanthus sp.	5'- acatggatccgctcagtcacaaag cacggt (SEQ ID No. 41) BamHI	5'-tagtactcgcgaggttgaactacat tcttatca (SEQ ID No. 42) XhoI
Discosoma sp. "red"	5'- acatggatccaggtcttccaagaat gttacc (SEQ ID No. 43) BamHI	5'-tagtactcgcaggagccaagttc agcctta (SEQ ID No. 44) XhoI
Discosoma striata	5'- acatggatccagttggtccaagagtgtg (SEQ ID No. 45) BamHI	5'-tagcgagctctatcatgcctc gtcacct (SEQ ID No. 46) SacI
Anemonia sulcata	5'- acatggatccgcttcttttaagaagact (SEQ ID No. 47) BamHI	5'-tagtactcgcagtccttgggagc ggcttg (SEQ ID No. 48) XhoI
Discosoma sp. "magenta"	5'- acatggatccagttgtccaagaatgtgat (SEQ ID No. 49) BamHI	5'-tagtactcgcaggccattacg ctaacc (SEQ ID No. 50) XhoI
Discosoma sp. "green"	5'-acatggatccagtgaccttaagaagaagaatg (SEQ ID No. 51)	5'-tagtactcgcagattcggtttaat gccttg (SEQ ID No. 52)

EXAMPLE 7**Novel Fluorescent Proteins and cDNAs Encoding the Proteins**

Seven cDNA full-length cDNAs encoding fluorescent
5 proteins were obtained (SEQ ID Nos. 45-51), and seven novel
fluorescent proteins were produced (SEQ ID Nos. 53-59). The spectral
properties of the isolated novel fluorescent proteins are shown in Table
7, and the emission and excitation spectra for the novel proteins are
shown in Figures 3-11.

10

TABLE 7Spectral Properties of the Isolated NFPs.

Species	NFP Name	Abs. Max. n m	Emission Maximum n m	Maximum Extinction Coeff.	Relative Quantum Yield*	Relative Brightness **
Anemonia majano	amFP486	458	486	40,000	0.3	0.43
Clavularia sp.	cFP484	456	484	35,300	0.6	0.77
Zoanthus sp.	zFP506	496	506	35,600	0.79	1.02
Zoanthus sp.	zFP538	528	538	20,200	0.52	0.38
Discosoma sp. "red"	drFP583	558	583	22,500	0.29	0.24
Discosoma striata	dsFP483	443	483	23,900	0.57	0.50
Anemonia sulcata	asFP600	572	596	56,200	<0.001	-
Discosoma sp "green"	dgFP512	502	512	20,360	0.3	0.21
Discosoma sp. "magenta"	dmFP592	573	593	21,800	0.11	0.09

5 *relative quantum yield was determined as compared to the quantum yield of *A. victoria* GFP.

**relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

Multiple alignment of fluorescent proteins is shown in Figure 2A. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP, SEQ ID No. 54). The amino acid sequences of the novel fluorescent proteins are labeled as SEQ ID Nos. 55-63. Two proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming β -sheets are underlined; the residues whose side chains form the interior of the β -can are shaded. Figure 2B shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

The following references were cited herein.

1. Ormo et al., (1996) Science 273: 1392-1395.
- 15 2. Yang, F., et al., (1996) Nature Biotech 14: 1246-1251.
3. Cormack, et al., (1996) Gene 173, 33-38.
4. Haas, et al., (1996) Current Biology 6, 315-324.
5. Yang, et al., (1996) Nucleic Acids Research 24, 4592-4593.
6. Ghoda, et al., (1990) J. Biol. Chem. 265: 11823-11826.
- 20 7. Prasher D.C. et al. (1992) Gene 111:229-33.
8. Kain et al. (1995) Biotechniques 19(4):650-55.
9. Chomczynski P., et al., (1987) Anal. Biochem. 162, 156-159.
10. Frohman et al., (1998) PNAS USA, 85, 8998-9002.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, 5 molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope 10 of the claims.

WHAT IS CLAIMED IS:

1. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

5 screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

10

2. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence hybridizes to a primer
15 selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

3. A method of analyzing a fluorescent protein in a cell,
20 comprising the steps of:

a) expressing a nucleic acid sequence encoding a fluorescent protein in said cell, wherein said protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63;
and

25 b) measuring a fluorescence signal from said protein.

4. The method of claim 3, further comprising the step of:
sorting said cell according to said signal.

5. The method of claim 4, wherein said step of sorting comprises sorting said cell by fluorescence activated cell sorting.

5 6. The method of claim 3, wherein said nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to said fluorescent protein, wherein said protein of interest is distinct from said fluorescent protein.

10 7. The method of claim 6, wherein the fluorescence signal indicates a presence of said gene of interest in said cell.

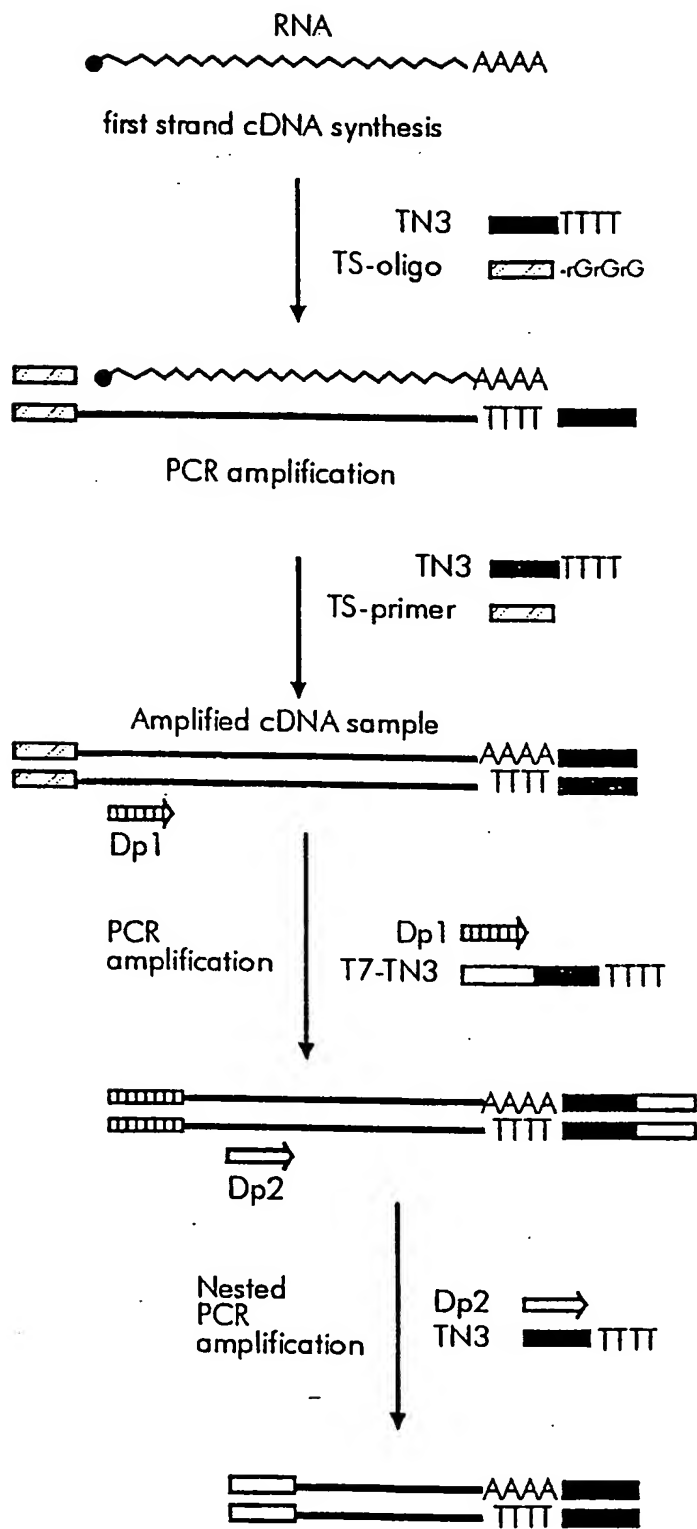
8. The method of claim 7, wherein said cell further comprises a protein of interest fused to said fluorescent protein.

15

9. The method of claim 8, further comprising the step of:

identifying an intracellular location of said fluorescent protein, thereby identifying an intracellular location of said protein of
20 interest.

10. An isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.



10	20	30	40	50	SEQ ID#
MSKGEELFTG.VVPILVELDGDVNGHKFSVSGEGEGDATYGKLT	TKLFICTT.GKLPVP..W	GFP			54
MAQSKHGLTK.EMTMKYRMEGCV	DGHKFEVITGEGIGYPFKGKQAINLCVV..EGGPLPFAE	zFP506			57
--H--KE.-----H-----N-----	-----T-----I-----S-	zFP538			58
MSWSKSVIKE.EMLIDLHLEGT	FNGHYFEIKGKGKGPNEGNTNTVLT	LEV..KGGPLPFGW			59
....M-AL--.Y-K-N-TM--VV--LP-K-R-D-----YQ-SQEL--T-V..-----SY		dgFP512			62
-RS--N----.F-RFKVRM--V---E---E-E-E-R-Y--H---K-K--..-----A-		drFP583			60
M-C--N----.F-RFKVRM--V---E---E-E-E-R-Y--HCS-K-M--..-----AF		dmFP592			63
...MASFLKK.TMPFKTTIEGT	VNGHYFKCTGKGEGNPFEGTQEMKIEVI..EGGPLPFAF	asFP600			61
MALSNKFIGD.DMKMTYHMDGCV	NHGYFTVKGEGNGKPYEGTQTSTFKVTMANGGPLAFSF	amFP486			55
◀KALTTMGVIKPDMKIKLKM	MEGNVNGHAFVIEGEGEGKPYDGTHTLNLEVKMAEGAPLPFSY	cFP484			56
60	70	80	90	100	110
PTLVTTFSYGVQCFSRYPDHMKQHDFK	SAM...:PEGYVQERTIFFKDDGNYKTRA	EVKFEGD..			GFP
DILSAAFNYGNRVFTEYPQDIV..DYFKNSC...	PAGYTWD	RSFLFEDGAVCICNADITVSVEEN			zFP506
-----G-K--D-I-----	-----G-----V-----K--				zFP538
HILCPQFQYGNKAFVHHPPDDIP..DYLKLSF...	PEGYTWERSMHFEDGGLCCITNDISLTGN..				dsFP483
D--TTM-----R---NY-E---..-IF-QTC	SGPNG--S-Q-T-TY---V-TA-SN--VV-D..				dgFP512
D--S-----S-VY-K--A---..-K-----FK--V-N---	VTV-Q-S--QDG..				drFP583
D--S-----S-VY-K--A---..-K-----FK--V-N---	VTVSQ-S--KDG..				dmFP592
HILSTSCMYGSKTFIKYVSGIP..DYFKQSF...	PEGFTWERTTTIEDGGFLTAHQDTS	LDGD..			asFP600
DILSTVFKYGNRCFTAYPTSMP..DYFKQAF...	PDGMSYERTFTYEDGGVATASWEISL	KGN..			amFP486
DILSNAFYQGNRALTKYPDDIA..DYFKQSF...	PEGYSWERTMTFEDKGIVKVKS	DISMEED..			cFP484
120	130	140	150	160	170
TLVNRIELKGI	DFKEDGNILGHKLEYNYN	SHNVYIMADKOKNGIKVNF	KIRHNI	EDGSVQL	
CMYHESKIFYGVNFPADGPVM.KKMTDNWEP	SCEKII	PVPKQGI	LKGDVSMYLL	KDGGRI	
-I--K-I-N-M-----	T--A--M-----				Y-
CENYDIKFTGLNFPNGPVV.QKKTG	WEPSTERLYP..RDGVLIGDIH	HALTVEGGGHYV			dsFP483
T-----H-M-A---LD--MM.--R-MK-----IMFE		---L-R-D-AMS-LLK----			R
--I-KV--I-V---SD--M.---M--A-----		---K-E--K--KLKD----			L
--I-EV--I-V---SD--M.-RR-R-----S-----		---K-----M--RL-----			L
CLVYKVKILGNFPADGPVM.QNKAGRWE	PATEIVYE..VDGVLRGQSLMALKCPGGRH	LT			asFP600
CFEHKSTFHGVNFPADGPVM.AKKTG	WDPSFEKMTV.:CDGILKGDVTAFLMLQGGGNYR				amFP486
SFIYEIRFDGMNFPNGPVM.QKKT	LKWEPTSEIMYV..RDGVLVGDISHSLLEGGGHYR				cFP484
180	190	200	210	220	230
ADHYQONTPIGDG.PVLLPD	NHYLSTOSALSKOPNEKR	DHMLLEFVTAAGITHGMD	ELYK		
CQFDTVYKAKSV..PRKMPDWHF	IQHKLTR	EDRS	DAKNQKWLTEHAIASGSALP		
-----S---E-----L-----			Q-----FP---A		
CDIKTVYPAKK...PVKMPGYHYVD	TKLVIRSNDKEFM.KVEEHEIAVARHHPLQSO				dsFP483
--FE-I-KPN- V----	D--F--HYIE-T-QQNYN V--LT-V-E--YSS-EKIGKSKA				dgFP512
VEF-SI-M---..--QL---Y---S--D-T-HNEDYT.I--QY-RTEG---	LFL				drFP583
VEF-SI-MV-- PS-QL---Y---S--DMT-HNEDYT V--QY-KTQ-----	FIKPLQ				dmFP592
CHLHTTYRSKKPASALKMPGFHFEDHRIE	IMEEVEKKGK.CYKQYEA	AVGRYCDAA	PSKLGHN		asFP600
CQFHTSYKTKK...PVTMPPNHVVEHRIART	DLDKGGN.SVQLTEHAVA	HITSVF	PF		amFP486
CDFKSIYKAKK...VVKLPDYHEVDHRIE	ILNHDKDYN.KVTLYENAVARYS	LLPSQA			cFP484

FIG. 2A

MKCKFVFECLSFVLAITNANI FLRNEADLEEKTLRIP

FIG. 2B

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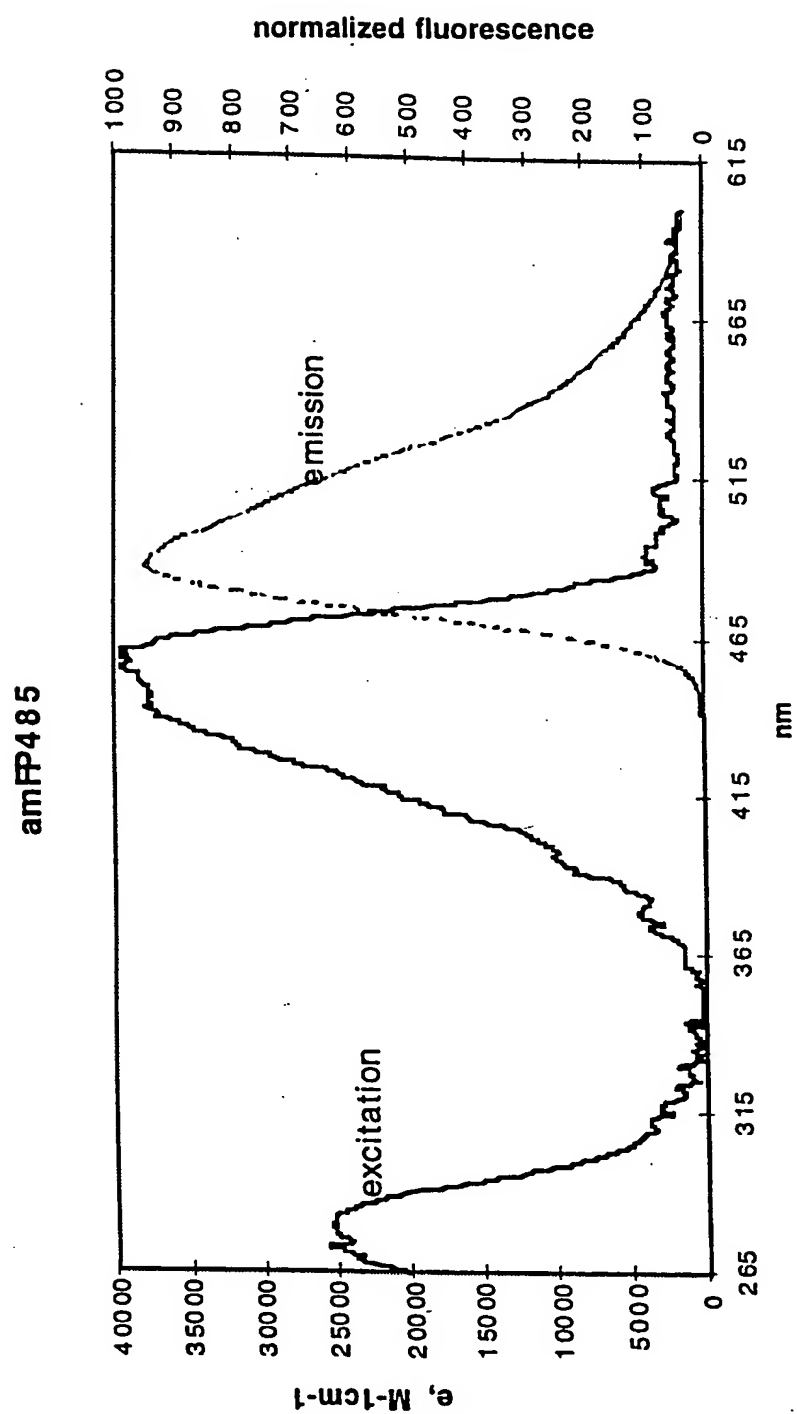


FIG. 3

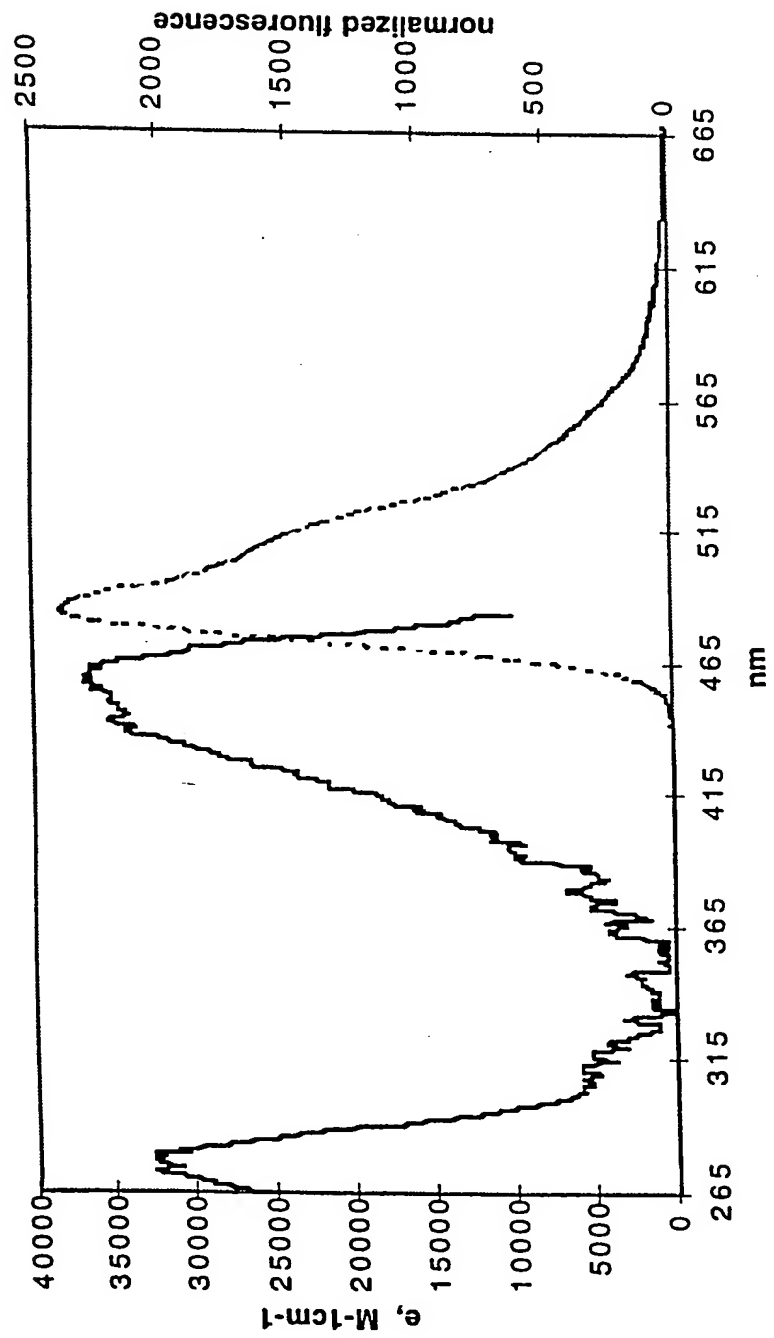
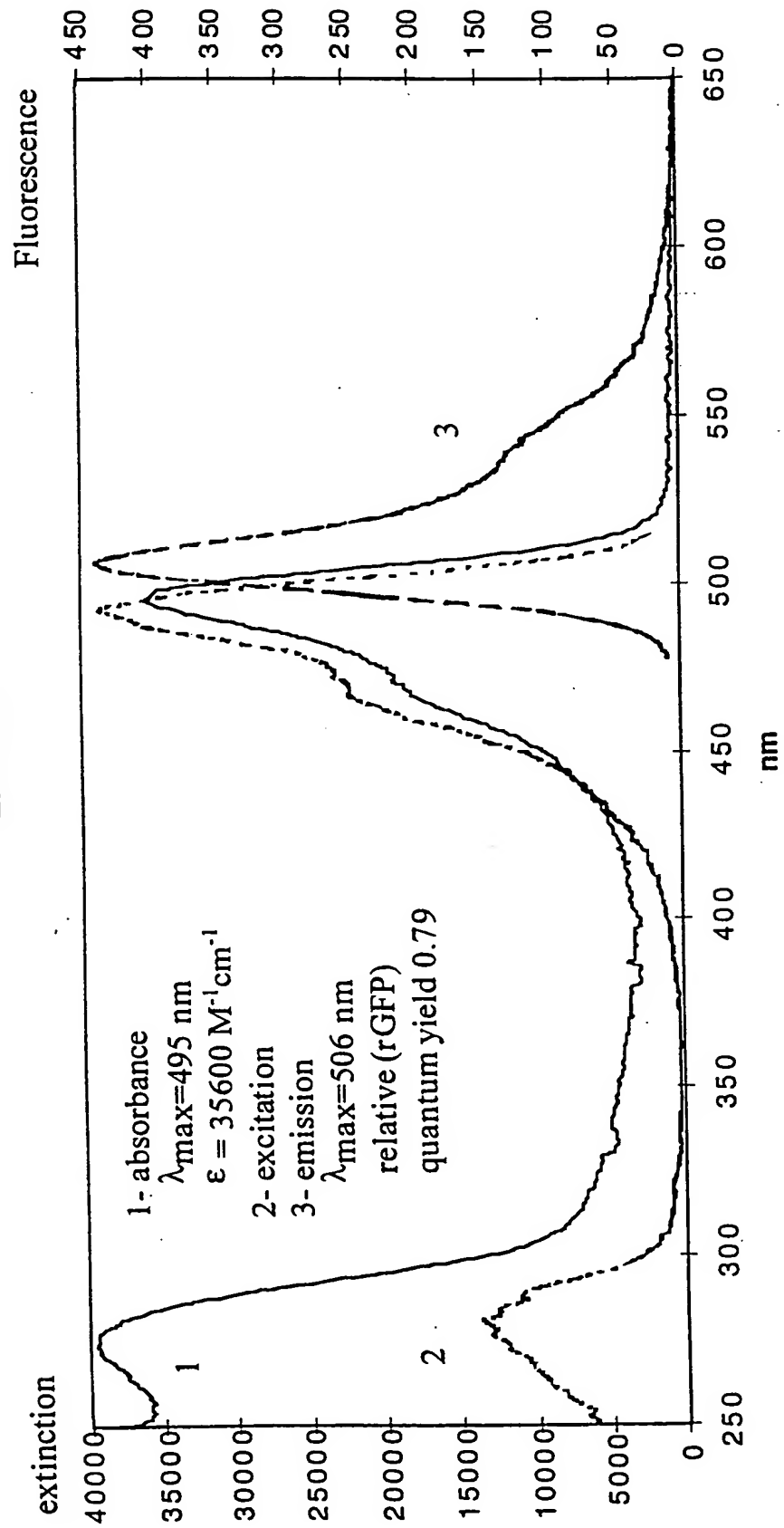


FIG. 4

zFP506

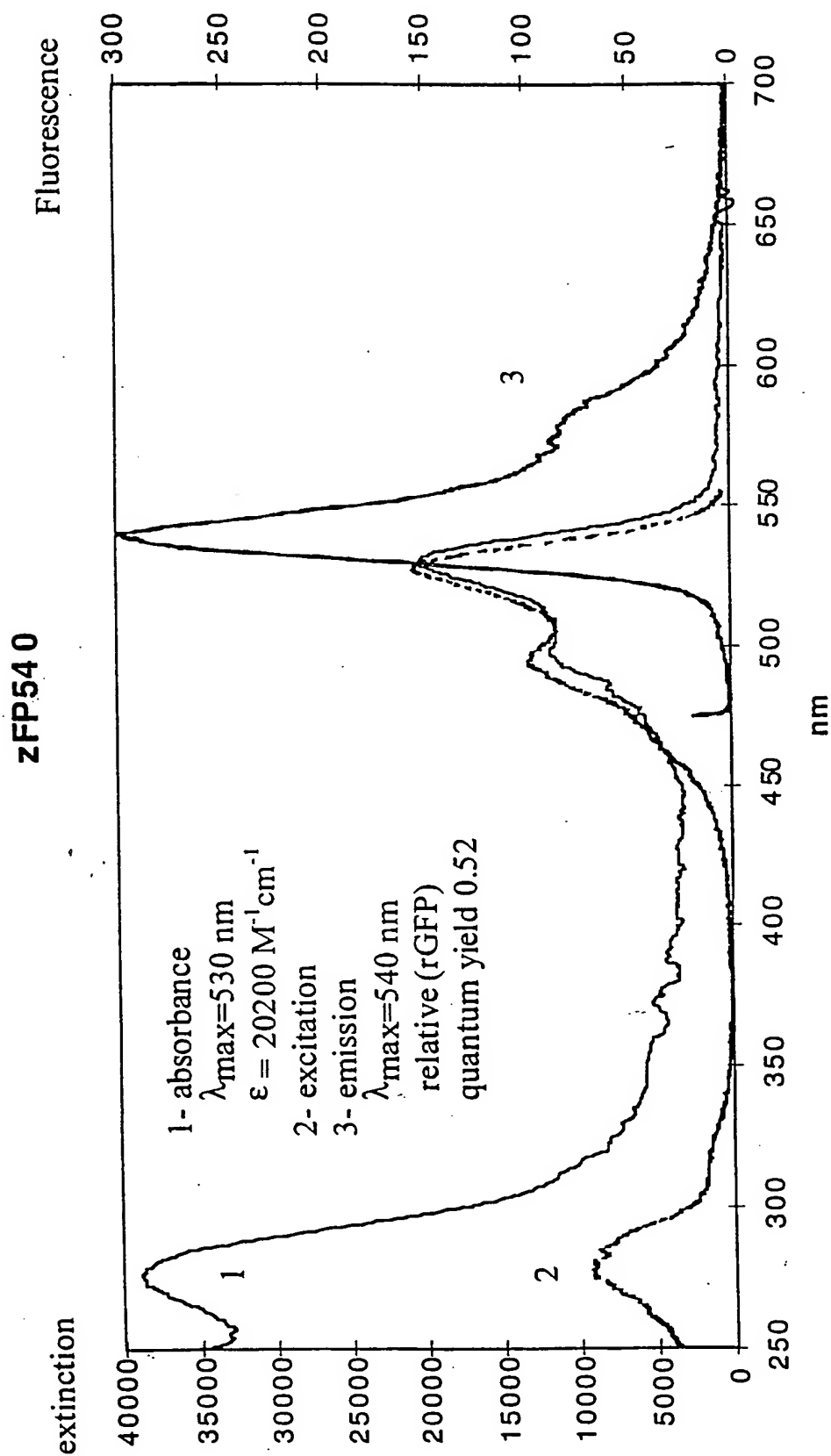


FIG. 6

dsFP484

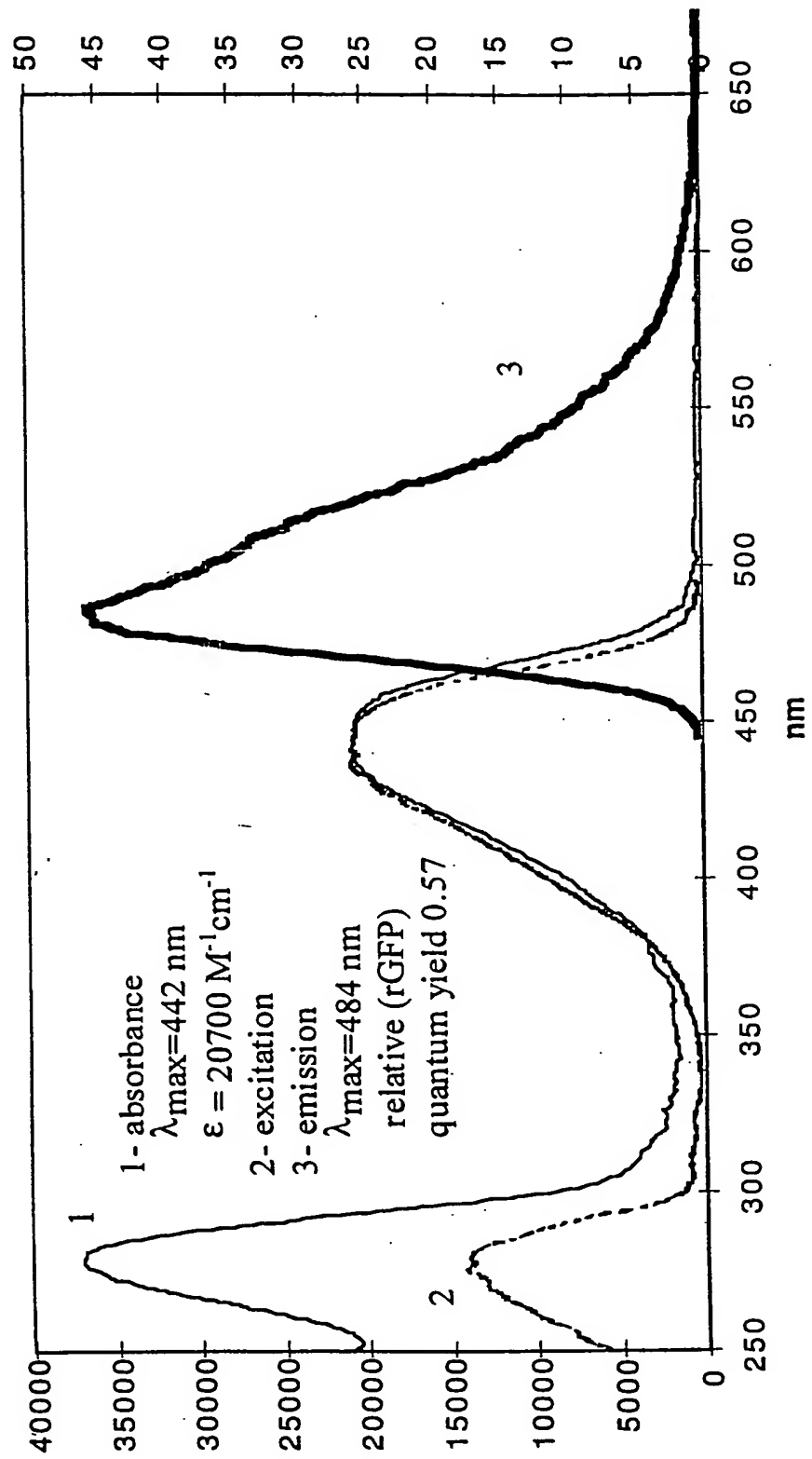


FIG. 7

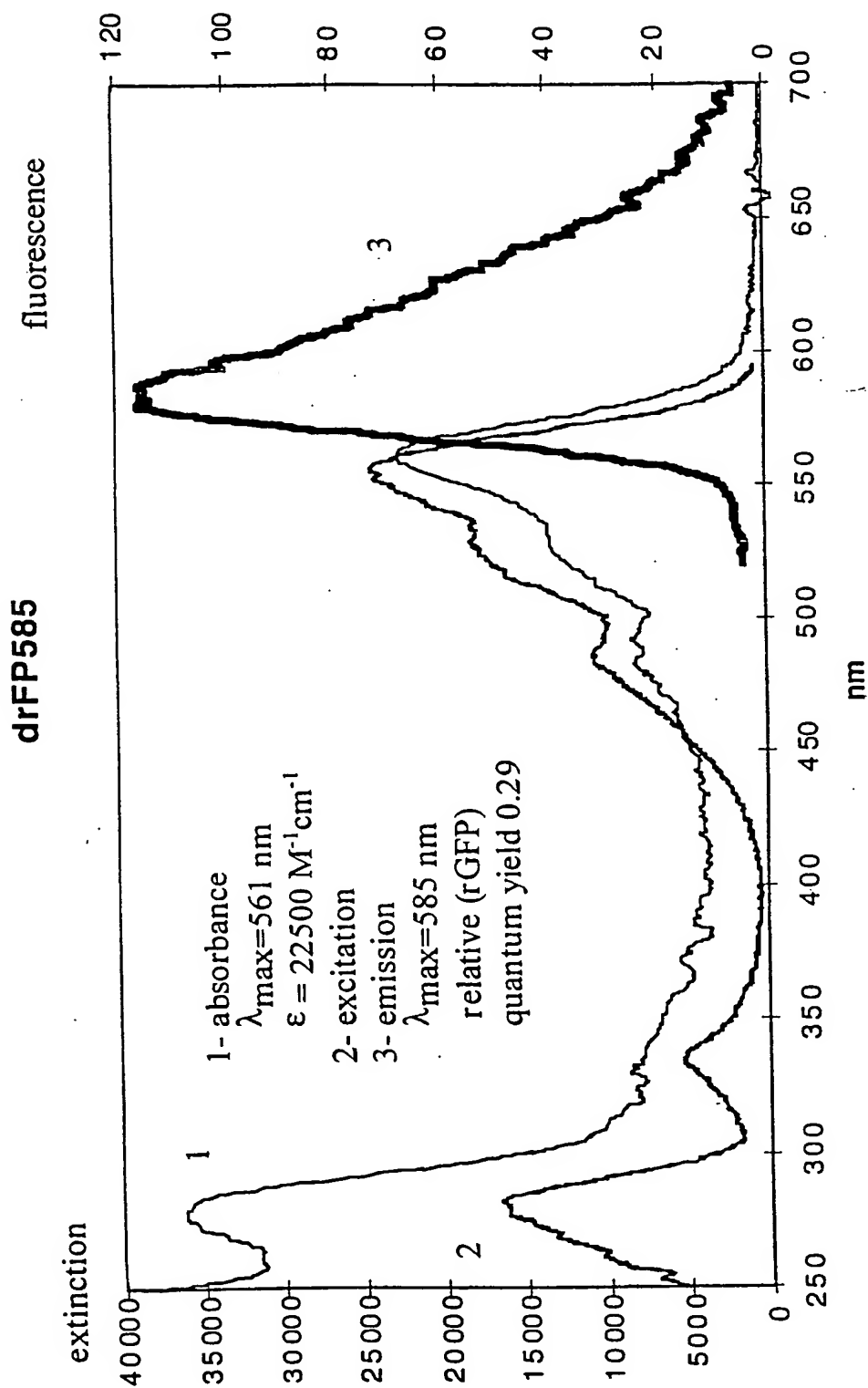


FIG. 8

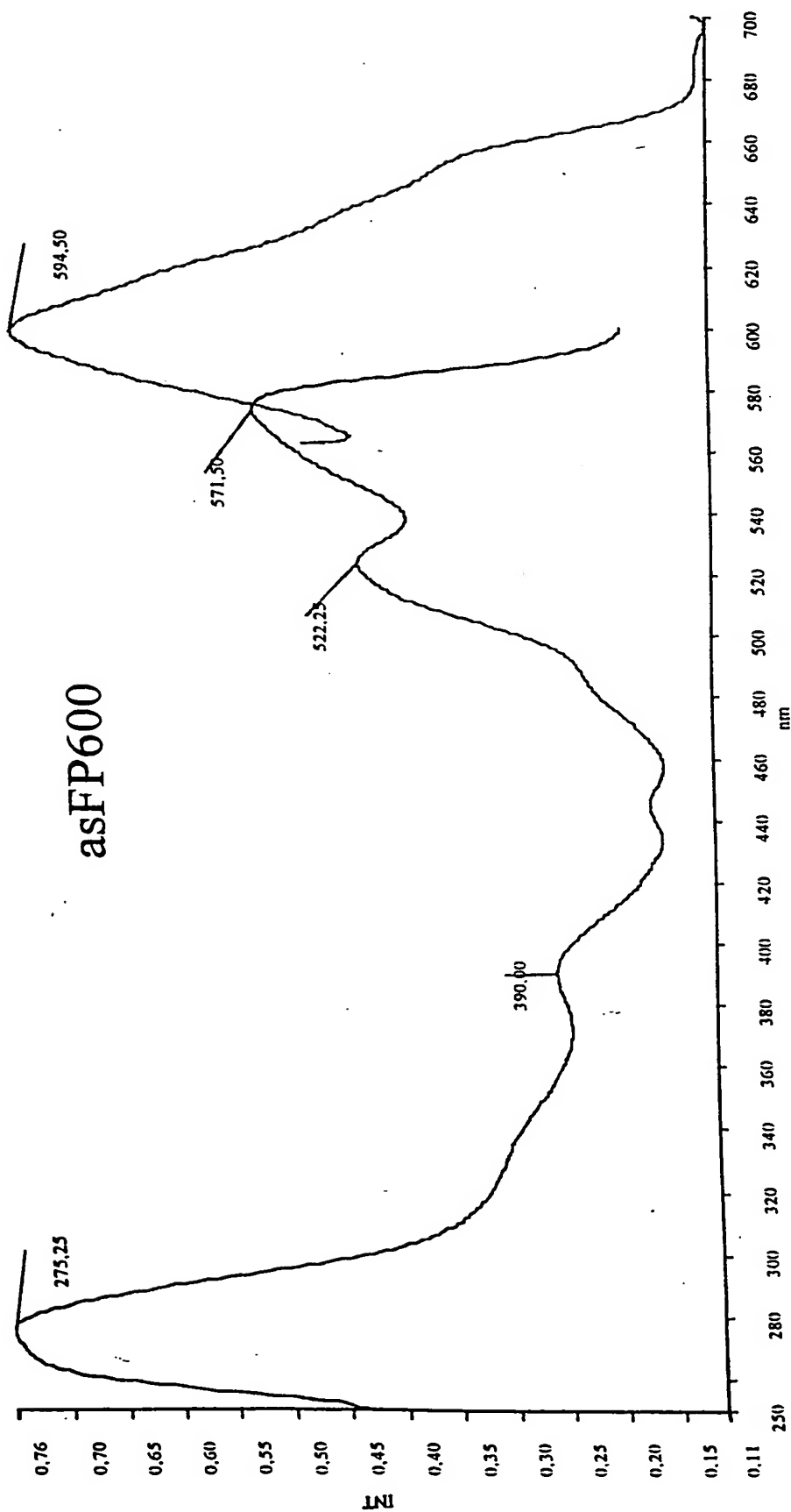


FIG. 9

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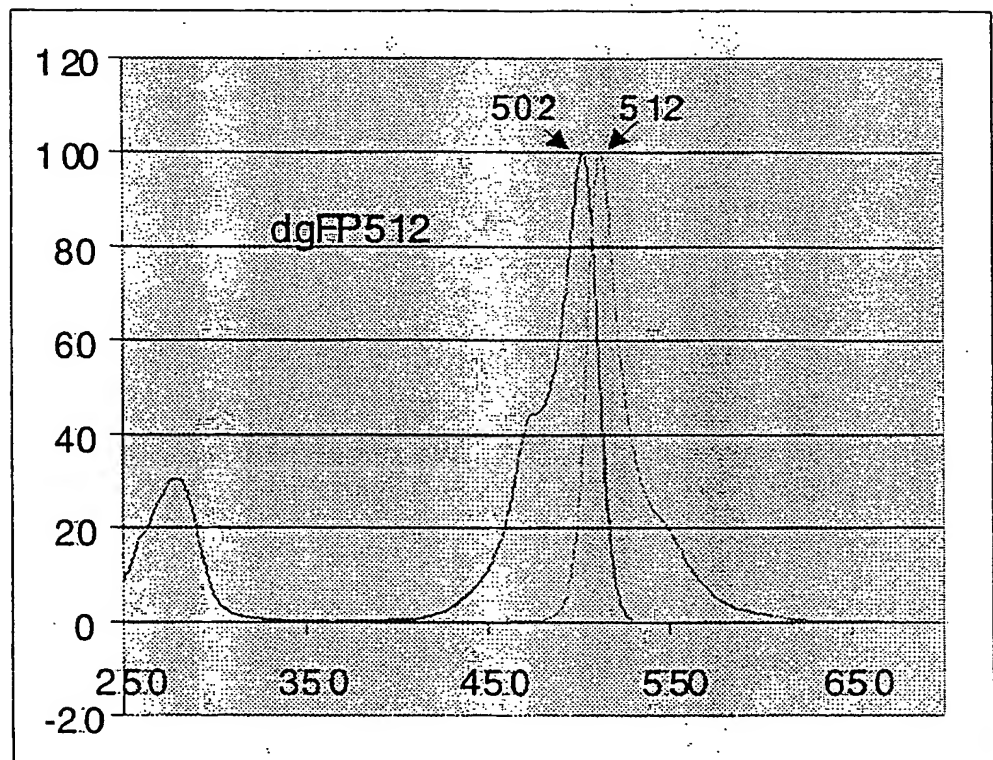


Fig. 10

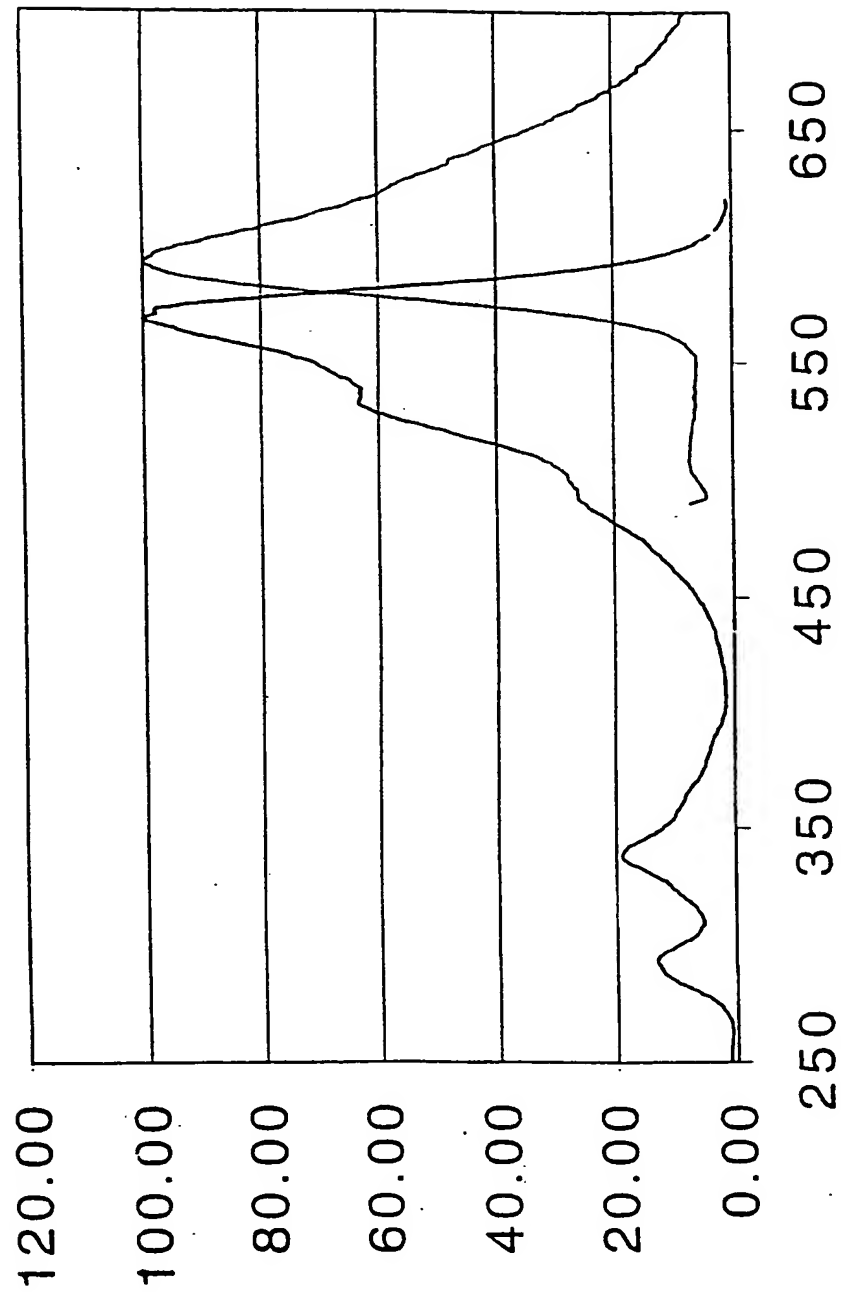


FIG. 11

SEQUENCE LISTING

<110> Lukyanov, Sergey A.
Labas, Yulii A.
Matz, Mikhail V.
5 Fradkov, Arcady F.
<120> Fluorescent proteins from non-bioluminescent
species of Class Anthozoa, genes encoding such
proteins and uses thereof
<130> D6196PCT
10 <141> 1999-12-10
<150> 09/210,330
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<211> 6
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 5
 10
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 20 protein; n at position 12 represents any of the
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 5
 35
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20
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 5

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 30 *Anemonia majano*
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Discosoma striata

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19

5 <210> 29

<211> 17

<212> DNA

<213> artificial sequence

<220>

10 <221> primer_bind

<223> gene-specific primer used for 5'-RACE for
Discosoma striata

<400> 29

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17

15

<210> 30

<211> 20

<212> DNA

<213> artificial sequence

20 <220>

<221> primer_bind

<223> gene-specific primer used for 5'-RACE for
Anemonia sulcata

<400> 30

25 cctctatctt catttcctgc

20

<210> 31

<211> 20

<212> DNA

30 <213> artificial sequence

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<221> primer_bind

<223> gene-specific primer used for 5'-RACE for
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25

30

22

35

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 10 <210> 36
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 15 <223> upstream primer used to obtain full coding region
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 20 <210> 37
 <211> 34
 <212> DNA
 <213> artificial sequence
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 25 <221> primer_bind
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 <210> 38
 <211> 29
 <212> DNA
 <213> artificial sequence
 35 <220>
 <221> primer_bind
 <223> upstream primer used to obtain full coding region

of nFPs from *Clavularia sp.*

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29

5 <210> 39

<211> 28

<212> DNA

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10 <221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Clavularia sp.*

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<211> 31

<212> DNA

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<221> primer_bind

<223> downstream primer used to obtain full coding
region of nFPs from *Clavularia sp.*

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31

<210> 41

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<212> DNA

30 <213> artificial sequence

<220>

<221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Zoanthus sp.*

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28

<210> 42
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 <212> DNA
 5 <213> artificial sequence
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 <210> 43
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 15 <212> DNA
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 <223> upstream primer used to obtain full coding region
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 30 <223> downstream primer used to obtain full coding
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<210> 46
10 <211> 28
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15 <223> downstream primer used to obtain full coding
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20 <211> 31
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of nFPs from *Anemonia sulcata*
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<223> downstream primer used to obtain full coding

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<400> 48

tagtactcga gtccttggga gcggttg 28

5 <210> 49

<211> 30

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<220>

10 <221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Discosoma sp. "magenta"*

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<211> 26

<212> DNA

<213> artificial sequence

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<223> downstream primer used to obtain full coding
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<210> 51

<211> 31

<212> DNA

30 <213> artificial sequence

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<223> upstream primer used to obtain full coding region
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 5 <213> artificial sequence
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 10 <400> 52

tagtactcga gattcgggtt aatgccttg 29

<210> 53
 <211> 33
 15 <212> DNA
 <213> artificial sequence
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 <223> TS-oligo used in cDNA synthesis and RACE
 20 <400> 53

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<210> 54
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 25 <212> PRT
 <213> *Aequorea victoria*
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 30 Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 5 10 15
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser
 20 25 30
 Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys
 35 35 40 45
 Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu
 50 55 60

SEQ 18/28

	Phe	Lys	Val	Thr	Met	Ala	Asn	Gly	Gly	Pro	Leu	Ala	Phe	Ser	Phe
					50					55					60
	Asp	Ile	Leu	Ser	Thr	Val	Phe	Lys	Tyr	Gly	Asn	Arg	Cys	Phe	Thr
					65					70					75
5	Ala	Tyr	Pro	Thr	Ser	Met	Pro	Asp	Tyr	Phe	Lys	Gln	Ala	Phe	Pro
					80					85					90
	Asp	Gly	Met	Ser	Tyr	Glu	Arg	Thr	Phe	Thr	Tyr	Glu	Asp	Gly	Gly
					95					100					105
	Val	Ala	Thr	Ala	Ser	Trp	Glu	Ile	Ser	Leu	Lys	Gly	Asn	Cys	Phe
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	Glu	His	Lys	Ser	Thr	Phe	His	Gly	Val	Asn	Phe	Pro	Ala	Asp	Gly
					125					130					135
	Pro	Val	Met	Ala	Lys	Lys	Thr	Thr	Gly	Trp	Asp	Pro	Ser	Phe	Glu
					140					145					150
15	Lys	Met	Thr	Val	Cys	Asp	Gly	Ile	Leu	Lys	Gly	Asp	Val	Thr	Ala
					155					160					165
	Phe	Leu	Met	Leu	Gln	Gly	Gly	Gly	Asn	Tyr	Arg	Cys	Gln	Phe	His
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	Thr	Ser	Tyr	Lys	Thr	Lys	Lys	Pro	Val	Thr	Met	Pro	Pro	Asn	His
20					185					190					195
	Val	Val	Glu	His	Arg	Ile	Ala	Arg	Thr	Asp	Leu	Asp	Lys	Gly	Gly
					200					205					210
	Asn	Ser	Val	Gln	Leu	Thr	Glu	His	Ala	Val	Ala	His	Ile	Thr	Ser
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					5					10					15
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		20		25		30
	Glu Lys Thr Phe	Arg Ile Pro Lys Ala	Leu Thr Thr Met Gly Val			
		35	40	45		
	Ile Lys Pro Asp	Met Lys Ile Lys Leu	Lys Met Glu Gly Asn Val			
5		50	55	60		
	Asn Gly His Ala	Phe Val Ile Glu Gly	Glu Gly Glu Gly Lys Pro			
		65	70	75		
	Tyr Asp Gly Thr	His Thr Leu Asn Leu	Glu Val Lys Glu Gly Ala			
		80	85	90		
10	Pro Leu Pro Phe	Ser Tyr Asp Ile Leu	Ser Asn Ala Phe Gln Tyr			
		95	100	105		
	Gly Asn Arg Ala	Leu Thr Lys Tyr Pro	Asp Asp Ile Ala Asp Tyr			
		110	115	120		
	Phe Lys Gln Ser	Phe Pro Glu Gly Tyr	Ser Trp Glu Arg Thr Met			
15		125	130	135		
	Thr Phe Glu Asp	Lys Gly Ile Val Lys	Val Lys Ser Asp Ile Ser			
		140	145	150		
	Met Glu Glu Asp	Ser Phe Ile Tyr Glu	Ile Arg Phe Asp Gly Met			
		155	160	165		
20	Asp Phe Pro Pro	Asn Gly Pro Val Met	Gln Lys Lys Thr Leu Lys			
		170	175	180		
	Trp Glu Pro Ser	Thr Glu Ile Met Tyr	Val Arg Asp Gly Val Leu			
		185	190	195		
	Val Gly Asp Ile	Ser His Ser Leu Leu	Leu Glu Gly Gly Gly His			
25		200	205	210		
	Tyr Arg Cys Asp	Phe Lys Ser Ile Tyr	Lys Ala Lys Lys Val Val			
		215	220	225		
	Lys Leu Pro Asp	Tyr His Phe Val Asp	His Arg Ile Glu Ile Leu			
		230	235	240		
30	Asn His Asp Lys	Asp Tyr Asn Lys Val	Thr Leu Tyr Glu Asn Ala			
		245	250	255		
	Val Ala Arg Tyr	Ser Leu Leu Pro Ser	Gln Ala			
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	<211>	230				
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<213> *Zoanthus sp.*

<220>

<223> amino acid sequence of zFP506

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	Glu	Gly	Ile	Gly	Tyr	Pro	Phe	Lys	Gly	Lys	Gln	Ala	Ile	Asn	Leu	35	40	45
10	Cys	Val	Val	Glu	Gly	Gly	Pro	Leu	Pro	Phe	Ala	Glu	Asp	Ile	Leu	50	55	60
	Ser	Ala	Ala	Phe	Asn	Tyr	Gly	Asn	Arg	Val	Phe	Thr	Glu	Tyr	Pro	65	70	75
15	Gln	Asp	Ile	Val	Asp	Tyr	Phe	Lys	Asn	Ser	Cys	Pro	Ala	Gly	Tyr	80	85	90
	Thr	Trp	Asp	Arg	Ser	Phe	Leu	Phe	Glu	Asp	Gly	Ala	Val	Cys	Ile	95	100	105
	Cys	Asn	Ala	Asp	Ile	Thr	Val	Ser	Val	Glu	Glu	Asn	Cys	Met	Tyr	110	115	120
20	His	Glu	Ser	Lys	Phe	Tyr	Gly	Val	Asn	Phe	Pro	Ala	Asp	Gly	Pro	125	130	135
	Val	Met	Lys	Lys	Met	Thr	Asp	Asn	Trp	Glu	Pro	Ser	Cys	Glu	Lys	140	145	150
25	Ile	Ile	Pro	Val	Pro	Lys	Gln	Gly	Ile	Leu	Lys	Gly	Asp	Val	Ser	155	160	165
	Met	Tyr	Leu	Leu	Leu	Lys	Asp	Gly	Gly	Arg	Leu	Arg	Cys	Gln	Phe	170	175	180
	Asp	Thr	Val	Tyr	Lys	Ala	Lys	Ser	Val	Pro	Arg	Lys	Met	Pro	Asp	185	190	195
30	Trp	His	Phe	Ile	Gln	His	Lys	Leu	Thr	Arg	Glu	Asp	Arg	Ser	Asp	200	205	210
	Ala	Lys	Asn	Gln	Lys	Trp	His	Leu	Thr	Glu	His	Ala	Ile	Ala	Ser	215	220	225
35	Gly	Ser	Ala	Leu	Pro											230		

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 5 <213> *Zoanthus sp.*
 <220>
 <223> amino acid sequence of zFP538
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 Tyr His Met Glu Gly Cys Val Asn Gly His Lys Phe Val Ile Thr
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 Gly Glu Gly Ile Gly Tyr Pro Phe Lys Gly Lys Gln Thr Ile Asn
 35 40 45
 15 Leu Cys Val Ile Glu Gly Gly Pro Leu Pro Phe Ser Glu Asp Ile
 50 55 60
 Leu Ser Ala Gly Phe Lys Tyr Gly Asp Arg Ile Phe Thr Glu Tyr
 65 70 75
 Pro Gln Asp Ile Val Asp Tyr Phe Lys Asn Ser Cys Pro Ala Gly
 20 80 85 90
 Tyr Thr Trp Gly Ser Phe Leu Phe Glu Asp Gly Ala Val Cys Ile
 95 100 105
 Cys Asn Val Asp Ile Thr Val Ser Val Lys Glu Asn Cys Ile Tyr
 110 115 120
 25 His Lys Ser Ile Phe Asn Gly Met Asn Phe Pro Ala Asp Gly Pro
 125 130 135
 Val Met Lys Lys Met Thr Thr Asn Trp Glu Ala Ser Cys Glu Lys
 140 145 150
 Ile Met Pro Val Pro Lys Gln Gly Ile Leu Lys Gly Asp Val Ser
 30 155 160 165
 Met Tyr Leu Leu Leu Lys Asp Gly Gly Arg Tyr Arg Cys Gln Phe
 170 175 180
 Asp Thr Val Tyr Lys Ala Lys Ser Val Pro Ser Lys Met Pro Glu
 185 190 195
 35 Trp His Phe Ile Gln His Lys Leu Leu Arg Glu Asp Arg Ser Asp
 200 205 210
 Ala Lys Asn Gln Lys Trp Gln Leu Thr Glu His Ala Ile Ala Phe
 215 220 225

Pro Ser Ala Leu Ala

230

5 <210> 59
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 <212> PRT
 <213> *Discosoma striata*
 <220>
 10 <223> amino acid sequence of dsFP483
 <400> 59

Met Ser Cys Ser Lys Ser Val Ile Lys Glu Glu Met Leu Ile Asp
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 Leu His Leu Glu Gly Thr Phe Asn Gly His Tyr Phe Glu Ile Lys
 15 20 25 30
 Gly Lys Gly Lys Gly Gln Pro Asn Glu Gly Thr Asn Thr Val Thr
 35 40 45
 Leu Glu Val Thr Lys Gly Gly Pro Leu Pro Phe Gly Trp His Ile
 50 55 60
 20 Leu Cys Pro Gln Phe Gln Tyr Gly Asn Lys Ala Phe Val His His
 65 70 75
 Pro Asp Asn Ile His Asp Tyr Leu Lys Leu Ser Phe Pro Glu Gly
 80 85 90
 Tyr Thr Trp Glu Arg Ser Met His Phe Glu Asp Gly Gly Leu Cys
 25 95 100 105
 Cys Ile Thr Asn Asp Ile Ser Leu Thr Gly Asn Cys Phe Tyr Tyr
 110 115 120
 Asp Ile Lys Phe Thr Gly Leu Asn Phe Pro Pro Asn Gly Pro Val
 125 130 135
 30 Val Gln Lys Lys Thr Thr Gly Trp Glu Pro Ser Thr Glu Arg Leu
 140 145 150
 Tyr Pro Arg Asp Gly Val Leu Ile Gly Asp Ile His His Ala Leu
 155 160 165
 Thr Val Glu Gly Gly Gly His Tyr Ala Cys Asp Ile Lys Thr Val
 35 170 175 180
 Tyr Arg Ala Lys Lys Ala Ala Leu Lys Met Pro Gly Tyr His Tyr
 185 190 195

	Val	Asp	Thr	Lys	Leu	Val	Ile	Trp	Asn	Asn	Asp	Lys	Glu	Phe	Met
					200					205					210
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					230										
					<210>					60					
					<211>					225					
10					<212>					PRT					
					<213>					<i>Discosoma</i> sp. "red"					
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	Gly	Glu	Gly	Glu	Gly	Arg	Pro	Tyr	Glu	Gly	His	Asn	Thr	Val	Lys
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	Leu	Lys	Val	Thr	Lys	Gly	Gly	Pro	Leu	Pro	Phe	Ala	Trp	Asp	Ile
					50					55					60
	Leu	Ser	Pro	Gln	Phe	Gln	Tyr	Gly	Ser	Lys	Val	Tyr	Val	Lys	His
					65					70					75
25	Pro	Ala	Asp	Ile	Pro	Asp	Tyr	Lys	Lys	Leu	Ser	Phe	Pro	Glu	Gly
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	Phe	Lys	Trp	Glu	Arg	Val	Met	Asn	Phe	Glu	Asp	Gly	Gly	Val	Val
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	Thr	Val	Thr	Gln	Asp	Ser	Ser	Leu	Gln	Asp	Gly	Cys	Phe	Ile	Tyr
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	Lys	Val	Lys	Phe	Ile	Gly	Val	Asn	Phe	Pro	Ser	Asp	Gly	Pro	Val
					125					130					135
	Met	Gln	Lys	Lys	Thr	Met	Gly	Trp	Glu	Ala	Ser	Thr	Glu	Arg	Leu
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35	Tyr	Pro	Arg	Asp	Gly	Val	Leu	Lys	Gly	Glu	Ile	His	Lys	Ala	Leu
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	Lys	Leu	Lys	Asp	Gly	Gly	His	Tyr	Leu	Val	Glu	Phe	Lys	Ser	Ile
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	Tyr	Met	Ala	Lys	Lys	Pro	Val	Gln	Leu	Pro	Gly	Tyr	Tyr	Tyr	Val
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	Asp	Ser	Lys	Leu	Asp	Ile	Thr	Ser	His	Asn	Glu	Asp	Tyr	Thr	Ile
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					<211>										232
10					<212>										PRT
					<213>										<i>Anemonia sulcata</i>
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	Glu	Gly	Asn	Pro	Phe	Glu	Gly	Thr	Gln	Glu	Met	Lys	Ile	Glu	Val
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	Ile	Glu	Gly	Gly	Pro	Leu	Pro	Phe	Ala	Phe	His	Ile	Leu	Ser	Thr
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	Ser	Cys	Met	Tyr	Gly	Ser	Lys	Thr	Phe	Ile	Lys	Tyr	Val	Ser	Gly
					65					70					75
25	Ile	Pro	Asp	Tyr	Phe	Lys	Gln	Ser	Phe	Pro	Glu	Gly	Phe	Thr	Trp
					80					85					90
	Glu	Arg	Thr	Thr	Thr	Tyr	Glu	Asp	Gly	Gly	Phe	Leu	Thr	Ala	His
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	Gln	Asp	Thr	Ser	Leu	Asp	Gly	Asp	Cys	Leu	Val	Tyr	Lys	Val	Lys
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	Ile	Leu	Gly	Asn	Asn	Phe	Pro	Ala	Asp	Gly	Pro	Val	Met	Gln	Asn
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	Lys	Ala	Gly	Arg	Trp	Glu	Pro	Ala	Thr	Glu	Ile	Val	Tyr	Glu	Val
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35	Asp	Gly	Val	Leu	Arg	Gly	Gln	Ser	Leu	Met	Ala	Leu	Lys	Cys	Pro
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	Gly	Gly	Arg	His	Leu	Thr	Cys	His	Leu	His	Thr	Thr	Tyr	Arg	Ser
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Lys Lys Pro Ala Ser Ala Leu Lys Met Pro Gly Phe His Phe Glu
 185 190 195
 Asp His Arg Ile Glu Ile Met Glu Glu Val Glu Lys Gly Lys Cys
 200 205 210
 5 Tyr Lys Gln Tyr Glu Ala Ala Val Gly Arg Tyr Cys Asp Ala Ala
 215 220 225
 Pro Ser Lys Leu Gly His Asn
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10 <210> 62
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 <212> PRT
 <213> *Discosoma sp. "green"*
 <220>
 15 <223> amino acid sequence of dgFP512
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 20 20 25 30
 Gly Lys Pro Tyr Gln Gly Ser Gln Glu Leu Thr Leu Thr Val Val
 35 40 45
 Lys Gly Gly Pro Leu Pro Phe Ser Tyr Asp Ile Leu Thr Thr Met
 50 55 60
 25 Phe Gln Tyr Gly Asn Arg Ala Phe Val Asn Tyr Pro Glu Asp Ile
 65 70 75
 Pro Asp Ile Phe Lys Gln Thr Cys Ser Gly Pro Asn Gly Gly Tyr
 80 85 90
 Ser Trp Gln Arg Thr Met Thr Tyr Glu Asp Gly Gly Val Cys Thr
 30 95 100 105
 Ala Thr Ser Asn Ile Ser Val Val Gly Asp Thr Phe Asn Tyr Asp
 110 115 120

Ile His Phe Met Gly Ala Asn Phe Pro Leu Asp Gly Pro Val Met
 125 130 135
 Gln Lys Arg Thr Met Lys Trp Glu Pro Ser Thr Glu Ile Met Phe
 140 145 150
 5 Glu Arg Asp Gly Met Leu Arg Gly Asp Ile Ala Met Ser Leu Leu
 155 160 165
 Leu Lys Gly Gly Gly His Tyr Arg Cys Asp Phe Glu Thr Ile Tyr
 170 175 180
 Lys Pro Asn Lys Val Val Lys Met Pro Asp Tyr His Phe Val Asp
 10 185 190 195
 His Cys Ile Glu Ile Thr Ser Gln Gln Asp Tyr Tyr Asn Val Val
 200 205 210
 Glu Leu Thr Glu Val Ala Glu Ala Arg Tyr Ser Ser Leu Glu Lys
 215 220 225
 15 Ile Gly Lys Ser Lys Ala
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 20 <212> PRT
 <213> *Discosoma sp. "magenta"*
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 Gly Glu Gly Glu Gly Arg Pro Tyr Glu Gly His Cys Ser Val Lys
 30 35 40 45

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5	Pro Ala Asp Ile Pro Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly	
	80	85 90
	Phe Lys Trp Glu Arg Val Met Asn Phe Glu Asp Gly Gly Val Val	
	100	105 110
	Thr Val Ser Gln Asp Ser Ser Leu Lys Asp Gly Cys Phe Ile Tyr	
10	115	120 125
	Glu Val Lys Phe Ile Gly Val Asn Phe Pro Ser Asp Gly Pro Val	
	130	135 140
	Met Gln Arg Arg Thr Arg Gly Trp Glu Ala Ser Ser Glu Arg Leu	
	145	150 155
15	Tyr Pro Arg Asp Gly Val Leu Lys Gly Asp Ile His Met Ala Leu	
	160	165 170
	Arg Leu Glu Gly Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile	
	175	180 185
	Tyr Met Val Lys Lys Pro Ser Val Gln Leu Pro Gly Tyr Tyr Tyr	
20	190	195 200
	Val Asp Ser Lys Leu Asp Met Thr Ser His Asn Glu Asp Tyr Thr	
	205	210 215
	Val Val Glu Gln Tyr Glu Lys Thr Gln Gly Arg His His Pro Phe	
	220	225 230
25	Ile Lys Pro Leu Gln	
	235	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29405

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C07K 14/435

US CL :435/6, 69.1; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 968; 530/350; 424/9.6, 436/172

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
***	The sequence diskette submitted with the description was defective; thus the references listed below were obtained solely by a WORD search, and not by a search of the SEQ ID NOs.	***
X, P	MATZ et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nature Biotechnology. October 1999, Volume 17, No. 10, pages 969-973, entire document.	1-10
X, P	DE 197 18 640 A1 (WIEDENMANN) 22 July 1999, entire document.	3-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

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Date of mailing of the international search report

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